THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 272, No. 7, Issue of February 14, pp. 4195–4200, 1997
Printed in U.S.A.

cGMP Stimulation of Cystic Fibrosis Transmembrane Conductance Regulator Cl^- Channels Co-expressed with cGMP-dependent Protein Kinase Type II but Not Type Iβ*

(Received for publication, October 2, 1996)

Arie B. Vaandrager‡§, Ben C. Tilly‡, Albert Smolenski¶, Sonja Schneider-Rasp¶, Alice G. M. Bot‡, Marcel Edixhoven‡, Bob J. Scholte‖, Thomas Jarchau¶, Ulrich Walter¶, Suzanne M. Lohmann¶, Wolfgang C. Poller¶, and Hugo R. de Jonge‡

From the ‡Department of Biochemistry, Cardiovascular Research Institute COEUR and the ‖Department of Clinical Genetics, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, 3000 DR Rotterdam, The Netherlands and ¶Laboratory of Clinical Biochemistry, Medical University Clinic Würzburg, 97080 Würzburg, Germany

In order to investigate the involvement of cGMP-dependent protein kinase (cGK) type II in cGMP-provoked intestinal Cl^- secretion, cGMP-dependent activation and phosphorylation of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels was analyzed after expression of cGK II or cGK Iβ in intact cells. An intestinal cell line which stably expresses CFTR (IEC-CF7) but contains no detectable endogenous cGK II was infected with a recombinant adenoviral vector containing the cGK II coding region (Ad-cGK II) resulting in co-expression of active cGK II. In these cells, CFTR was activated by membrane-permeant analogs of cGMP or by the cGMP-elevating hormone atrial natriuretic peptide as measured by $^{125}I^-$ efflux assays and whole-cell patch clamp analysis. In contrast, infection with recombinant adenoviruses expressing cGK Iβ or luciferase did not convey cGMP sensitivity to CFTR in IEC-CF7 cells. Concordant with the activation of CFTR by only cGK II, infection with Ad-cGK II but not Ad-cGK Iβ enabled cGMP analogs to increase CFTR phosphorylation in intact cells. These and other data provide evidence that endogenous cGK II is a key mediator of cGMP-provoked activation of CFTR in cells where both proteins are co-localized, *e.g.* intestinal epithelial cells. Furthermore, they demonstrate that neither the soluble cGK Iβ nor cAMP-dependent protein kinase are able to substitute for cGK II in this cGMP-regulated function.

In intestinal epithelium a cGMP-signaling pathway can activate cystic fibrosis transmembrane conductance regulator (CFTR)[1] Cl^- channels, resulting in the net secretion of salt and water (1, 2). Guanylin and/or uroguanylin, small peptides derived from larger precursor proteins synthesized by intestinal epithelial cells, may function as the physiological activator of the cGMP-mediated signaling route in intestine by activating guanylyl cyclase C located in the apical membrane of enterocytes (2–4). Heat-stable enterotoxins secreted by various pathogenic strains of *Escherichia coli* mimic the action of guanylin and elicit a severe secretory diarrhea by hyperactivating guanylyl cyclase C (2–4).

Localization studies have suggested a key role for a recently cloned isotype of cGMP-dependent protein kinase (cGK), designated type II, as the mediator of the cGMP-provoked intestinal Cl^- secretion (5–7). Type II cGK is expressed predominantly in epithelial cells of the intestine (5–7), although it was also detected in kidney (6, 8, 9) and brain (6, 8, 10). In contrast, type I cGK, consisting of α and β isoforms, and shown to act as a key regulator of cardiovascular homeostasis (11, 12), is not expressed in enterocytes (7). Furthermore, purified endogenous pig cGK II, in contrast to bovine lung cGK I, was shown to activate CFTR in excised membrane patches (13). However, the mechanism of the apparent cGK isotype selectivity in activating CFTR was not clear, since both cGK I and cGK II could phosphorylate immunoprecipitated CFTR *in vitro* (13). An explanation for this discrepancy might be that cGK II but not cGK I selectively phosphorylates CFTR in a native environment. To examine this possibility we investigated activation and phosphorylation of CFTR in intact cells expressing either cGK II or cGK Iβ. Endogenous expression of cGK isoforms in intact cells also permitted use of native enzyme in these experiments such that alterations, particularly of cGK II, that occur during purification could be avoided. Purification of the membrane-bound cGK II requires the use and subsequent removal of detergents, a procedure which potentially contributes to nonspecific (hydrophobic) interactions of this enzyme. Furthermore, purification results in partial proteolytic modification of cGK II and renders cGK II less sensitive to cGMP (5, 13).

For the analyses of the interactions of CFTR and cGK II or cGK Iβ in intact cells, we established a highly efficient co-expression system in which rat intestinal IEC-CF7 cells previously stably transfected with CFTR (14) were infected with recombinant adenoviral vectors containing the cDNA of cGK II or cGK Iβ. Here we report that co-expression with cGK II but not with cGK Iβ renders CFTR sensitive to activation by cGMP in intact cells. Furthermore, CFTR is shown to be a selective substrate for only cGK II-mediated phosphorylation under physiological conditions, providing a possible explanation for the present and previously (13) observed isotype-specific activation of CFTR by cGK II.

EXPERIMENTAL PROCEDURES

Materials—Protein A-Sepharose was from Pierce, 3-isobutyl-1-methylxanthine (IBMX), and rat atrial natriuretic peptide (ANP) from Sigma, cGMP analogs from Biolog (Bremen, Germany), and

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* This work was supported by the Netherlands Organization for Scientific Research (NWO) and the Deutsche Forschungsgemeinschaft (SFB 355). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. Biochemistry, Medical Faculty, Erasmus University Rotterdam, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands. Tel.: 31-10-408-1111; Fax: 31-10-436-0615.

[1] The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; cGK, cGMP-dependent protein kinase, cAK, cAMP-dependent protein kinase; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-cGMP; 8-Br-PET-cGMP, β-phenyl-1,N^2-etheno-8-bromo-cGMP; ANP, atrial natriuretic peptide.

[^{32}P]orthophosphate, [γ-^{32}P]ATP, $^{125}I^-$, and the enhanced chemiluminescence (ECL) system from Amersham Corp. Polyclonal cGK II or cGK I antibodies raised against recombinant cGK II or cGK Iβ expressed in *E. coli* were prepared as described (7). The polyclonal antibody C449 against CFTR (15) was a gift from A. C. Nairn (Rockefeller University, New York, NY). The cGK substrate peptide 2A3 (RRKVSKQE) and the Walsh inhibitor peptide (PKI-(5–24)amide) were synthesized by D. Palm (University of Würzburg, Germany).

Construction of Adenovirus Vectors Expressing cGK Iβ and cGK II—For the development of the recombinant virus Ad-cGKII, the coding sequence of rat cGK II (6) was cloned into the multiple cloning site between the CMV promoter and the bGH-3′ untranslated region of the adenoviral transfer plasmid pZS2 (16). Upstream of the expression cassette the plasmid also contains 0.5 kilobase 5′ terminal sequence of wild-type adenovirus type 5. The recombinant plasmid cGKII/pZS2 was linearized with *Xba*I and ligated with the long *Xba*I fragment of the DNA of RR5, an Ad5 mutant carrying a unique *Xba*I site and a deletion of the E1 region ranging from nucleotide position 445–3333 (16). The ligation product was then transfected into the E1a-transformed human embryonic kidney cell line 293, and after 10–14 days, recombinant virus was screened for by polymerase chain reaction, recovered, and plaque-purified as described previously (16). For the construction of the recombinant virus Ad-cGK Iβ, the expression cassette from pCl (an expression vector obtained from Promega containing the CMV promoter and the SV40 poly(A)) was cloned into the multiple cloning site of the adenoviral transfer plasmid pΔE1sp1A (a plasmid containing N-terminal E1-deleted sequences of Ad5, obtained from Microbix Biosystems lnc., Toronto, Canada) to yield the recombinant plasmid pCMVI. Then the coding sequence of human cGKIβ (17, 18) was cloned into the expression cassette of pCMVI resulting in the plasmid cGKIβ/pCMVI. Recombinant adenovirus was generated via homologous recombination between cGKIβ/pCMVI and pJM17 (Microbix), a bacterial plasmid containing full-length Ad5 DNA, following cotransfection of both plasmids into 293 cells (19). The titer of the adenoviral preparations was approximately 1 plaque-forming unit per 500 particles.

Culture and Infection of IEC-CF7 Cells—Rat intestine-derived IEC-6 cells stably expressing CFTR (IEC-CF7) were prepared as described (14) and cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 0.1 IU/ml insulin, 0.2 mg/ml G418, 0.1 mg/ml streptomycin, and 0.04 mg/ml penicillin. Two days after plating, confluent (subconfluent in the case of patch clamp analysis) monolayers of cells were infected by replacing the medium with fresh medium additionally containing the adenovirus vectors (usually 5 × 10^9 particles/ml).

Protein Kinase Assays and Immunoblotting—Two days after infection cells were washed twice with ice-cold phosphate-buffered saline, scraped with a rubber policeman in buffer A (150 mM NaCl, 10 mM $NaPO_4$ pH 7.4, 1 mM EDTA, 100 μg/ml trypsin inhibitor, and 20 μg/ml leupeptin) and homogenized by brief sonication (three bursts of 3 s, peak-to-peak amplitude 15–20 μm). Cytosol and membranes were separated by centrifugation at 150,000 × *g* for 60 min at 4 °C in an Airfuge. Protein kinase activity was determined by incubation of the samples (10 μg of protein) at 30 °C for 4 min in 40 μl of 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM 3-isobutyl-1-methylxanthine, 25 mM Na-β-glycerophosphate, 200 nM protein kinase A inhibitor (PKI), 0.1 mg/ml of a cGK substrate peptide 2A3 (RRKVSKQE; see Ref. 18), 1 μCi of [γ-^{32}P]ATP, 300 μM unlabeled ATP and cGMP or cGMP analogs as described (20). Immunoblotting was performed as described earlier (21). Immunoreactive proteins were detected after incubation with cGK II or cGK I antibody (1:3000) by the enhanced chemiluminescence method and quantitated by the Molecular Imaging System GS-363 (Bio-Rad) using standards of purified bovine lung cGK I, recombinant rat intestine cGK II expressed in and purified from Sf9 cells (22), or rat intestinal brush border membranes containing an established amount of endogenous cGK II.

$^{125}I^-$ Efflux Studies—Cells which had been infected 2 days earlier were loaded with tracer for 1.5 h under a humidified 95% air, 5% CO_2 atmosphere at 37 °C in a modified Meyler solution (108 mM NaCl, 4.7 mM KCl, 1.3 mm $CaCl_2$, 1 mM $MgCl_2$, 0.8 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 20 mM $NaHCO_3$, 20 mM HEPES, and 10 mM glucose, pH 7.4) containing 5 μCi/ml $^{125}I^-$. After removal of extracellular isotope with three washes of 3 ml of modified Meyler solution, isotope efflux from the cells was determined at 37 °C by addition and consecutive replacements of 1 ml of modified Meyler solution at 1–3-min intervals and was expressed as fractional efflux per minute as described (23).

Whole-cell Patch Clamp Analysis—Whole-cell Cl^- currents were measured 1 or 2 days after viral infection using a Biologic RK-300 amplifier essentially as described (24). Signals were filtered at 1 kHz, digitized (Digidata 1200, Axon Instruments Inc., Foster City, CA), and analyzed using pClamp 6.0 software (Axon Instruments). Heat-polished patch pipettes (3–6 MΩ) filled with an intracellular solution containing 120 mM *N*-methyl-D-glucamine, 85 mM aspartic acid, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM MgATP, 5 mM *N*-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES; pH adjusted to 7.3 with HCl; [Cl^-], 43 mM) were used. The extracellular solution contained 140 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM glucose, and 10 mM TES (pH 7.3). Cells were clamped at a holding potential of 0 mV and membrane currents were measured during depolarizing and hyperpolarizing voltage steps (+50 to −90 mV in 20-mV decrements). All experiments were performed at 32 °C.

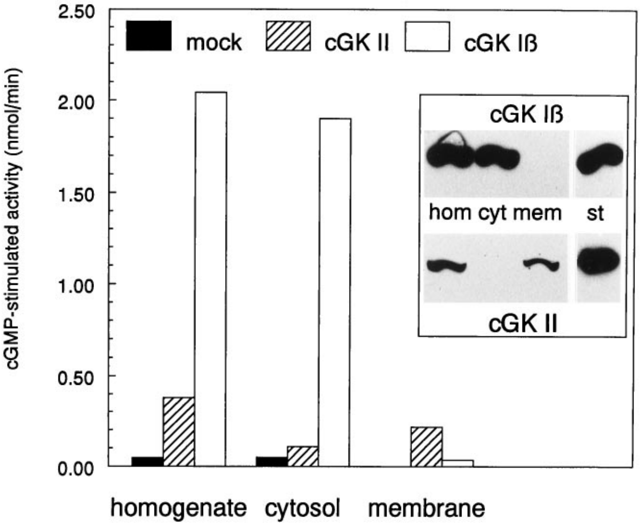

FIG. 1. **Adenovirus-mediated transfer of cGMP-dependent protein kinases (cGK) in IEC-CF7 cells.** Rat intestinal IEC-CF7 cells stably transfected with CFTR Cl^- channels were infected with 5 × 10^9 particles/ml (approximately 10^7 plaque-forming units/ml) of a replication-deficient adenovirus containing the cDNA of luciferase (*mock; solid bar*), cGK II (*hatched bar*), or cGK Iβ (*open bar*). Two days after infection, cells were harvested, homogenized, and separated into cytosol and membrane fractions. Phosphotransferase activity was determined with a cGK-selective substrate (2A3) in the presence or absence of 10 μM cGMP and was expressed per milligram of homogenate protein after correction for basal activity in the absence of cGMP. *Inset,* immunoblots of the homogenate (5 μg protein; *hom*), cytosol (*cyt*), and membrane (*mem*) fractions of cells infected with adenovirus containing cGK Iβ (*top*) or cGK II (*bottom*). The blots were labeled with the respective specific antibodies against cGK I and II. In the *lanes at right,* 10 ng of pure cGK I (*top*) or rat intestinal brush borders containing 10 ng of cGK II (*bottom*) were loaded as standards (*st*). Shown are results of a typical experiment which was performed three times.

Phosphate Labeling and Immunoprecipitation of CFTR—Two days after infection, confluent cells grown in 6-well plates (Costar; 10 cm^2/well) were incubated at 37 °C for 1 h in 1 ml of modified Meyler solution without phosphate supplemented with 0.5 mCi of [^{32}P]orthophosphate. Labeled cells were incubated for an additional 20 min with membrane-permeable cGMP analogs or forskolin in the same medium. Subsequently the medium was removed and the cells were lysed with 300 μl of 1% SDS containing 100 μg/ml leupeptin and 100 μM phenylmethylsulfonyl fluoride. The lysates were diluted 2-fold with a solution of 50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 10 mM EDTA, 200 mM NaF, 40 mM sodium pyrophosphate, and 5% Nonidet P-40 and were homogenized by six passages through a thin needle (0.5 × 16 mm). ^{32}P-Phosphorylated CFTR was immunoprecipitated with a purified anti-CFTR antibody C449 as described (15), separated by 6% SDS-polyacrylamide gel electrophoresis, visualized by autoradiography, and quantitated with the Molecular Imaging System GS-363.

RESULTS AND DISCUSSION

Adenovirus-mediated Transfer of cGK Iβ and cGK II—To study the interaction of CFTR with cGK I or II, we used rat intestinal IEC-CF7 cells stably transfected with CFTR Cl^- channels (14), which contain no detectable endogenous cGK I or cGK II by immunoblotting (data not shown), as well as extremely little soluble and no measurable membrane-associated

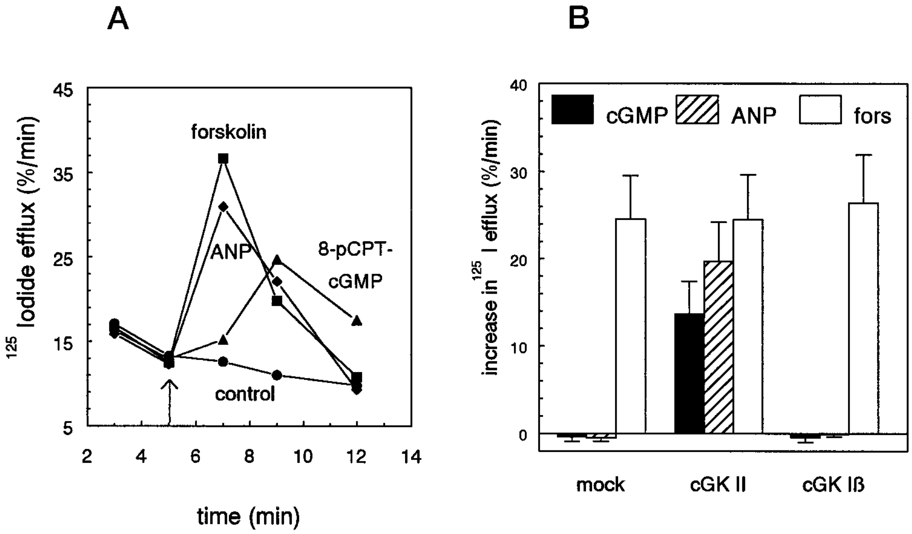

FIG. 2. **cGMP increases iodide efflux in IEC-CF7 cells expressing cGK II but not cGK Iβ.** Rat intestinal IEC-CF7 cells stably transfected with CFTR Cl⁻ channels were infected with replication deficient adenovirus (Ad) containing the cDNA of either luciferase (*mock*), cGK II, or cGK Iβ. Two days after infection, CFTR activity was monitored by measurements of fractional ^{125}I⁻ efflux. *A,* time course of ^{125}I⁻ efflux in Ad-cGK II-infected IEC-CF7 cells. At 5 min (*arrow*) 50 μM 8-pCPT-cGMP (▲), 0.1 μM ANP (♦), 10 μM forskolin (■), or vehicle (*control;* ●) was added to the efflux medium. *B,* maximal increment in ^{125}I⁻ efflux as determined 2 min after addition of forskolin (*fors; open bars*) and ANP (*hatched bars*) or 4 min after addition of cGMP analogs (*cGMP; solid bars*) to IEC-CF7 cells infected as shown. ^{125}I⁻ efflux was corrected for basal efflux at the same time points determined in the absence of the agonists. Concentration of the agonists added was as in *A*, except that 20 μM 8-Br-PET-cGMP was used to activate cGK Iβ. Data are means ± S.E. of 3–6 experiments.

cGMP-stimulated phosphotransferase activity (Fig. 1). Infection of IEC-CF7 cells with 5 × 10^9 particles of replication deficient adenovirus containing the cDNA of rat cGK II (Ad-cGK II) resulted in the expression of 0.5 ± 0.2 μg of cGK II/mg protein as assessed by immunoblotting ($n = 4$; Fig. 1), which is in the range of the endogenous cGK II content of isolated rat enterocytes determined by immunoblotting (0.2–0.4 μg/mg protein; data not shown). Furthermore, only the full-length 86-kDa form of cGK II was observed in homogenates of Ad-cGK II-infected IEC-CF7 cells (Fig. 1), which is similar to cGK II in native rat intestinal brush border membranes (Fig. 1) (7). In contrast, a mixture of cGK II forms (86 kDa intact and 70 and 75 kDa proteolyzed forms) was present in the purified preparations of pig cGK II used in previous experiments demonstrating cGK II-mediated activation of CFTR in excised membrane patches (5, 12). Infection of IEC-CF7 cells with a similar dose of adenovirus vector containing the coding region of human cGK Iβ (Ad-cGK Iβ) produced a relatively high expression level of cGK Iβ (2.4 ± 1 μg/mg protein; detected by immunoblotting, $n = 4$) corresponding to a large increase in phosphotransferase activity exceeding the activity measured for cGK II by 5-fold (Fig. 1). In contrast to cGK II, which was associated primarily with the membrane fraction, cGK Iβ was cytosolic (Fig. 1). The observed subcellular localization of the recombinant cGKs after adenovirus-mediated transfer are in agreement with the membrane localization of endogenous cGK II (5, 7) and the soluble character of endogenous cGK Iβ (25). The selectivities of the recombinant cGK II and cGK Iβ expressed in the IEC-CF7 cells for membrane permeant cGMP analogs were similar to those previously determined for endogenous or recombinant forms of cGK II and Iβ (18, 22, 26, 27),)[2] *i.e.* cGK II was preferentially activated by 8-pCPT-cGMP over 8-Br-PET-cGMP (K_a = 0.1 and 1.7 μM, respectively; data not shown), and cGK Iβ was more readily activated by 8-Br-PET-cGMP than by 8-pCPT-cGMP (K_a = 0.04 and 0.9 μM, respectively; data not shown). Taken together, these results show that both the amount and subcellular distribution of cGK isotype expression produced by adenovirus-mediated gene transfer in IEC-CF7 cells reflects that of endogenous cGK I and cGK II, allowing a meaningful comparison of the effects of recombinant cGK isotypes on CFTR in IEC-CF7 cells.

Activation of CFTR by cGMP in cGK II-expressing Cells—^{125}I⁻ efflux measurements provide a simple assay for monitoring the activation of CFTR Cl⁻ channels (14, 23). The cAMP-elevating agent forskolin caused a large increase in ^{125}I⁻ efflux from IEC-CF7 cells (Fig. 2) but not from CFTR-deficient wild-type or mock-transfected IEC-6 cells (14). In contrast, both the membrane-permeable cGMP analog 8-pCPT-cGMP and the cGMP-elevating hormone atrial natriuretic peptide (ANP) (28) were unable to mimic the forskolin-provoked increase in ^{125}I⁻ efflux in either IEC-CF7 cells infected with control adenovirus containing luciferase cDNA (Fig. 2*B*, *mock*), or in noninfected IEC-CF7 cells (data not shown). These results indicate that cGMP was unable to provoke activation of CFTR via endogenous cAMP-dependent protein kinase (cAK) in cGK-deficient IEC-CF7 cells under the conditions tested. However, after infection of IEC-CF7 cells with 5 × 10^9 particles/ml Ad-cGK II, addition of 8-pCPT-cGMP caused a gradual increase in the ^{125}I⁻ efflux rate, reaching a maximum after 4 min (14 ± 4%/min above basal, $n = 6$; Fig. 2). A 5-fold lower dose of Ad-cGK II (10^9 particles/ml) resulted in an approximately 5-fold lower expression of cGK II, and a 3–4-fold lower increment in ^{125}I⁻ efflux rate (3.8 ± 1.3%/min; $n = 3$) in response to 8-pCPT-cGMP (data not shown). Relatively high doses of Ad-cGK II (>2 × 10^{10} particles/ml) were more effective than the standard dose of 5 × 10^9 particles/ml in facilitating the 8-pCPT-cGMP-provoked increase in ^{125}I⁻ efflux but were also toxic for the IEC-CF7 cells as judged from their morphology and an increased rate of basal ^{125}I⁻ efflux (data not shown).

8-pCPT-cGMP was unable to stimulate ^{125}I⁻ efflux in CFTR-deficient IEC-6 cells infected with Ad-cGK II (data not shown), further strengthening the concept that the CFTR Cl⁻ channel is the mediator of cGMP/cGK II-enhanced ^{125}I⁻ efflux in IEC-CF7 cells. Accordingly, whole-cell patch clamp analysis of Ad-cGK II-infected IEC-CF7 cells stimulated with 8-pCPT-cGMP revealed rapid induction of a linear Cl⁻ current that was in-

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[2] A. B. Vaandrager, unpublished observations.

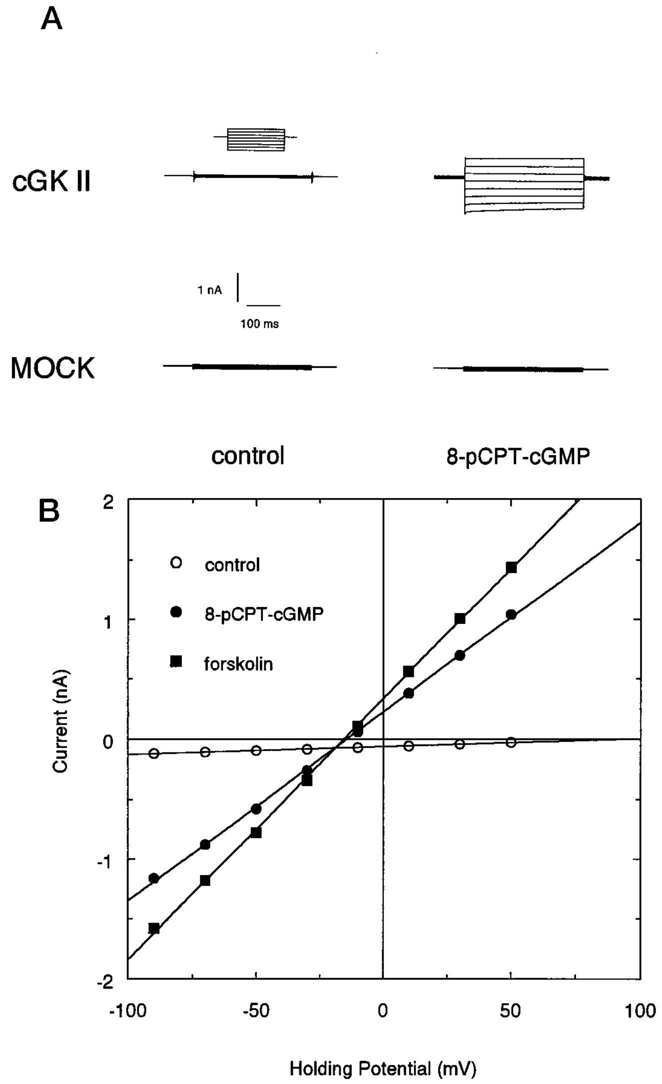

FIG. 3. **cGMP increases whole-cell chloride current in IEC-CF7 cells expressing cGK II.** Rat intestinal IEC-CF7 cells stably transfected with CFTR Cl^- channels were infected with replication-deficient adenovirus containing the cDNA of either cGK II or luciferase (*mock*). One or two days after infection, whole-cell Cl^- currents were measured. Cells were clamped at a holding potential of 0 mV, and membrane currents were recorded during depolarizing and hyperpolarizing voltage steps (+50 to −90 mV in 20-mV decrements; see *inset* above first trace). *A,* current traces from cGK II or mock-infected single cells stimulated with 8-pCPT-cGMP (50 μM) and subsequently forskolin (10 μM). Control traces represent basal currents prior to stimulation. *B,* current-to-voltage relationship of basal (○) membrane current and the currents provoked by 8-pCPT-cGMP (●) and forskolin (■) in the cGK II-infected cell shown in *A*.

distinguishable from forskolin-provoked currents (Fig. 3) (24). This 8-pCPT-cGMP-triggered anion current was observed in 8 of 9 IEC-CF7 cells infected with Ad-cGK II. In contrast, only in 1 of 6 mock-infected cells was a small increase observed, indicating that cGK II expression was a prerequisite for CFTR Cl^- channel activation.

In excised membrane patches, the activation of CFTR Cl^- channels by purified pig cGK II was relatively slow in comparison to their activation by cAK (13). In Ad-cGK II-infected IEC-CF7 cells 8-pCPT-cGMP caused a similar sluggish activation of CFTR (Fig. 2*A*). As ANP was shown previously to induce a prompt rise in intracellular cGMP in IEC cells by activating endogenous guanylyl cyclases (28), the time course of ANP activation of $^{125}I^-$ efflux was compared with the response to the cGMP analog. As shown in Fig. 2*A*, the rate of ANP-stimulated $^{125}I^-$ efflux was similar to that observed with the cAMP-elevating agonist forskolin, suggesting that cGK II and cAK are able to activate CFTR in intact cells with similar kinetics and that the lag phase in activation by 8-pCPT-cGMP is due to a relatively slow permeation of the analog across the IEC-CF7 cell membrane. These results also imply that the delayed CFTR channel opening in response to purified cGK II observed in excised patches (13) is apparently an *in vitro* artifact and may represent the lag time needed for re-anchoring of the solubilized enzyme to the membrane.

In agreement with our previous *in vitro* observations in excised membrane patches (13), cGMP activation of CFTR in intact IEC-CF7 cells was specifically mediated by type II cGK, since neither 8-Br-PET-cGMP nor ANP provoked an increase in $^{125}I^-$ efflux in Ad-cGK Iβ-infected cells (Fig. 2*B*). However, these cells showed a normal forskolin response, excluding any deleterious effect of cGK Iβ expression on CFTR activation (Fig. 2*B*). Furthermore, the ANP-provoked increase in cGMP did not differ between Ad-cGK II and Ad-cGK Iβ-infected IEC-CF7 cells (data not shown). Since infection of IEC-CF7 cells with equivalent doses of adenovirus caused a 5-fold higher expression of cGK Iβ than cGK II, and this dose of Ad-cGK Iβ did not stimulate $^{125}I^-$ efflux, whereas even a 5-fold lower dose of Ad-cGK II still did, we conclude that cGK II is at least 25-fold more effective than cGK Iβ in activating CFTR Cl^- channels. However, since both cGK II and cGK I were found to phosphorylate immunopurified CFTR and a cloned regulatory domain fragment of CFTR (CF-2) *in vitro* with similar kinetics (13), we next investigated the *in situ* phosphorylation of CFTR in cGK II and cGK Iβ-expressing IEC-CF7 cells.

Phosphorylation of CFTR in IEC-CF7 Cells—As shown in Fig. 4, 8-pCPT-cGMP caused an almost 3-fold increase in ^{32}P labeling of CFTR in Ad-cGK II-infected IEC-CF7 cells. In contrast, 8-Br-PET-cGMP had no effect on CFTR phosphorylation in Ad-cGK Iβ-infected cells, despite the fact that CFTR phosphorylation in response to forskolin was similar in both cGK Iβ and cGK II-expressing cells. Therefore, the increase in CFTR phosphoryl content paralleled the activation of CFTR-mediated $^{125}I^-$ efflux observed upon cGMP application. This suggests

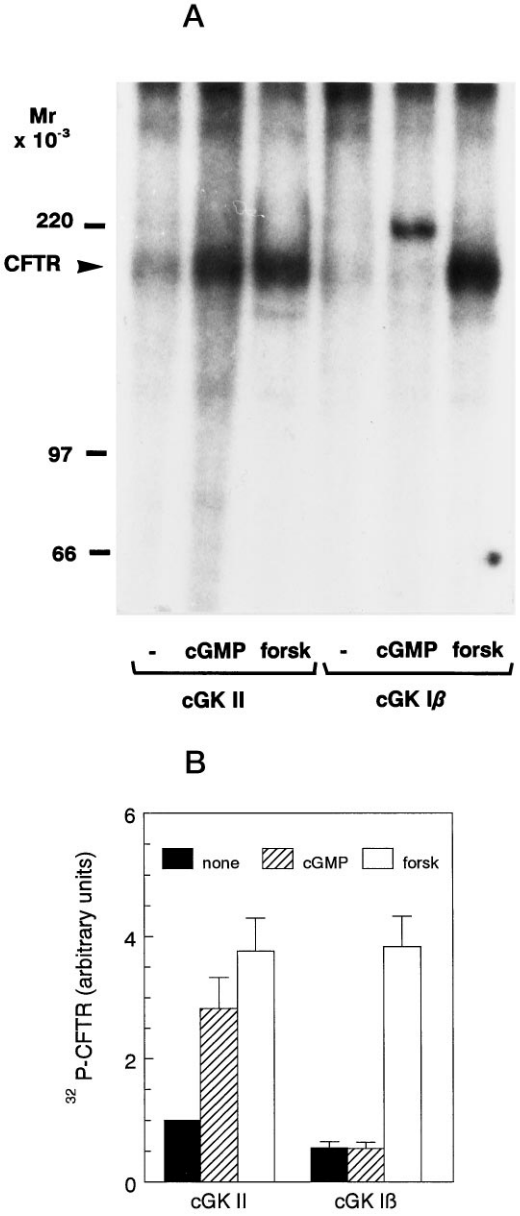

FIG. 4. **cGMP promotes phosphorylation of CFTR in IEC-CF7 cells expressing cGK II but not cGK Iβ.** Rat intestinal IEC-CF7 cells stably transfected with CFTR Cl^- channels were infected with replication-deficient adenovirus containing the cDNA of either cGK II or Iβ (5×10^9 particles/ml). Two days after infection, cells were metabolically labeled with inorganic ^{32}P for 1 h and subsequently incubated for 20 min with vehicle (−, *none*), 10 μM forskolin (*forsk*), or either 50 μM 8-pCPT-cGMP in the case of cGK II (*cGMP*) or 20 μM 8-Br-PET-cGMP in the case of cGK Iβ (*cGMP*). Subsequently CFTR was immunoprecipitated and separated by 6% SDS-polyacrylamide gel electrophoresis. *A,* autoradiograph showing ^{32}P-phosphorylated CFTR (30-day exposure). The position of CFTR as determined with *in vitro* ^{32}P-phosphorylated CFTR (see Refs. 13 and 14) is indicated with an *arrowhead.* The 220-kDa protein in *lane 5* is nonspecific, as it does not comigrate with CFTR and was not observed in other experiments. *B,* amount of ^{32}P incorporated into CFTR was quantitated by a phospho-imager and expressed relative to the basal ^{32}P incorporation into CFTR in Ad-cGK II-infected IEC-CF7 cells (*cGK II, none*). Data are means ± S.E. of three experiments.

that selective phosphorylation of CFTR, rather than subtle differences in the phosphorylation of individual sites within the CFTR molecule, may be the molecular basis for the present and previously (13) observed cGK II isotype-selective activation of CFTR. The cGK I-mediated phosphorylation of immunoprecipitated CFTR (13) may be another example of promiscuous substrate utilization inherent in *in vitro* phosphorylation studies. We might speculate that the different capabilities of the two cGK isotypes for CFTR phosphorylation in intact cells may be due to: (i) a potential difference in the structure of the regulatory domain of native CFTR in comparison to that of immunoprecipitated CFTR or (ii) a kinetic advantage of cGK II in phosphorylating native CFTR due to their co-localization in the same subcellular (membrane) compartment (20). Even a small kinetic advantage in phosphorylation may be important for tipping the balance between phosphorylation and dephosphorylation of CFTR in a native environment that may contain other regulatory components like phosphatases, which are absent in isolated preparations of CFTR or CF-2. Finally, since the *in vivo* phosphorylation data strictly do not distinguish whether CFTR is directly phosphorylated by cGK II or by another kinase, the remote possibility that the cGK II isotype-specific activation of CFTR is at the level of a regulator/cofactor endogenously expressed in IEC-CF7 cells cannot as yet be ruled out.

In conclusion, our demonstration that cGK II expression rendered CFTR sensitive to modulation by cGMP in cells which did not previously display a cGMP-inducible Cl^- conductance indicates that cGK II is a key mediator of cGMP-provoked activation of CFTR in, *e.g.*, intestinal epithelial cells where both proteins are co-localized (7). This conclusion is also corroborated by the previously observed correlation between the presence of cGK II and detection of cGMP-induced CFTR-mediated Cl^- secretion in different intestinal segments (7). Furthermore, the role of cGK II in mediating cGMP-provoked activation of CFTR activation cannot be mimicked by cGK I or cAK in our cell system. In particular, the ascribed role of cAK in the cGMP-induced activation of CFTR in several cell lines (29–33) may therefore not be widely valid but restricted to cells either expressing a type III cGMP-inhibited phosphodiesterase to elevate cAMP (29) or cells in which very high levels of cGMP can be attained to cross-activate cAK (30–32). The intestinal cell lines T84 and Caco-2 used to demonstrate the latter mechanism appear nevertheless to be unsuitable models for studying physiological mechanisms, since they do not contain detectable levels of cGK II or cGK I (7). Furthermore, we have shown (7) that the major localization of cGK I in smooth muscle cells of the villus lamina propria, in contrast to the epithelial brush border where cGK II is located, makes cGK I an unlikely endogenous mediator of cGMP effects on CFTR. The particularly intriguing aspect of our present results is the observation that even when present cGK I cannot substitute for cGK II in phosphorylating CFTR in intact cells or in stimulating CFTR-mediated Cl^- conductance. This not only reinforces the caveat regarding *in vitro* phosphorylation studies but, more importantly, may be a clue to mechanisms of subcellular compartmentalization of functions.

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